Journal of Medical Virology 55:321–327 (1998)

Retrospective Serological and Genetic Study of the Distribution of Hantaviruses in Greece

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A retrospective serological and genetic study of hantaviruses responsible for hemorrhagic fever with renal syndrome (HFRS) in Greece during the last 17 years is presented. Fifty-one serum samples taken from 30 HFRS cases previously diagnosed by immunofluorescence assay were tested by ELISA for IgG (Hantaan, Dobrava, and Puumala) and IgM antibodies (Hantaan and Puumala). Results were compatible with the majority of infections being related to hantaviruses carried by rodents of the subfamily Murinae. RNA was extracted from 26 selected samples and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers specifically designed for the detection of hantaviruses associated with murine (MS-N-specific, MM-G1-specific primers) or arvicoline rodents (PPT-N-specific primers). In addition, primers previously designed for the detection of the G2 coding region of the Murinae-associated hantaviruses were also used. Sequencing of the PCR products was then performed, followed by phylogenetic analysis of nucleotide sequence differences. Eleven out of the 26 serum samples tested were found to be positive by PCR with the MS-N primers, whereas four were positive with the MM-G1 primers, and only two with the G2 primers. None of the samples was found positive with the PPT primers. The sequence analysis showed that the virus that was responsible for these 11 HFRS cases was the Dobrava virus, which is endemic throughout the Balkans. ***J. Med. Virol. 55:321–327, 1998.***

KEY WORDS: hantavirus; hemorrhagic fever with renal syndrome; ELISA; RT-PCR

INTRODUCTION

Hantaviruses, members of the genus *Hantavirus* in the family Bunyaviridae, are enveloped, negative-stranded RNA viruses that cause two distinct diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Each serotype of these viruses has its natural reservoir, mainly a rodent species [Elliott, 1990]. HFRS, characterized by fever, renal failure, and, in some cases, hemorrhagic manifestations, is caused by Hantaan (HTN), Dobrava (DOB), Seoul (SEO), and Puumala (PUU) viruses carried by the striped field mouse (*Apodemus agrarius*), the yellow-necked field mouse (*Apodemus flavicollis*), rats (*Rattus norvegicus* and *R. rattus*), and the bank vole (*Clethrionomys glareolus*), respectively. HPS, which is characterized by fever, headache, myalgias, cough, and acute respiratory dysfunction [Duchin et al., 1994], is caused by Sin Nombre (SN) virus carried by the deer mouse, *Peromyscus maniculatus* [Nichol et al., 1993], and several additional recently discovered hantaviruses associated with other Sigmodontinae subfamily rodents (reviewed in Schmaljohn and Hjelle [1997]). HFRS usually occurs in Asia or Europe, while HPS occurs in the Americas. Several other hantaviruses, including Prospect Hill (PH), Thailand (THAI), Thottapalayam, Khabarovsk (KBR), and Tula (TUL), have been described, but they are not at present implicated with disease in humans.

In Greece, HFRS was first diagnosed in 1983. Since then, there have been approximately 200 cases described. In 1987, a HTN-like virus (Porogia) was reported to be isolated from the urine of a severely ill HFRS patient. Extensive serological cross-reactivity

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Accepted 18 March 1998

TABLE I. Description of HFRS Patient Samples and ELISA and PCR Results

Case	Location	Day/ month/ year	Time post onset[a]	IFAHTN IgM	HTN IgG[b]	DOB IgG	PUU IgG	HTN IgM	PUU IgM	MS-N[c]	MM-G1	PPT-N	G2 primers
KS	Hospital, C.G.	??-Mar-79	a	512	1.5	3.8	0.0	7.3	0.6				
NA	Promaxi, N.G.	20-Aug-84	a18d	1024	0.6	0.8	–0.1	5.6	1.1	Neg	Neg	Neg	Neg
NA	Promaxi, N.G.	22-Aug-84	a20d	512	0.8	1.4	–0.1	5.5	1.0				
CG	Ioannina, W.G.	29-Jul-85	a9d	1024	0.2	0.2	0.1	7.2	0.1	DOB	Neg		Neg
CG	Ioannina, W.G.	1-Aug-85	a15d	1024	0.1	–0.1	0.0	7.0	0.0				
CG	Ioannina, W.G.	4-Sep-86	c1y	256	2.1	0.6	0.0	6.1	0.4				
CG	Ioannina, W.G.	10-Apr-89	c3, 5y	Neg	3.7	3.7	2.3	0.1	0.0				
TI	Ioannina, W.G.	29-Jul-85	a12d	2048	0.7	0.8	0.0	6.1	0.3	DOB	Neg		Neg
TI	Ioannina, W.G.	31-Jul-85	a15d	2048	0.1	0.1	–0.1	0.0	0.0				
PE	Ioannina, W.G.	2-Aug-85	a16d	1024	2.1	1.5	–0.1	6.5	0.2	Neg	Neg		Neg
PE	Ioannina, W.G.	20-Mar-89	c3, 5y	Neg	4.9	6.3	2.1	0.1	0.0				
SI	Ioannina, W.G.	3-Sep-85	a10d	4096	0.6	0.9	0.0	7.1	0.3	DOB	Neg		Neg
TD	Poroia, N.G.	2-Apr-86	a10d	512	0.2	0.6	–0.1	1.7	1.8	DOB	Neg	Neg	Neg
NF	Ioannina, W.G.	23-Aug-86	a8d	512	1.4	0.4	0.0	6.7	0.6	DOB	Neg		Neg
NF	Ioannina, W.G.	30-Aug-86	a15d	512	1.5	0.2	0.1	5.4	0.4				
NF	Ioannina, W.G.	4-Sep-86	a20d	512	1.8	0.3	0.0	5.3	0.4				
NF	Ioannina, W.G.	28-Feb-89	c2y, 6m	Neg	5.2	3.8	1.2	0.7	0.1				
NF	Ioannina, W.G.	13-Apr-89	c2y, 8m	Neg	4.7	3.5	1.1	0.8	0.0				
KU	Ioannina, W.G.	??-??-87	a	256	3.3	5.3	1.3	0.4	0.0				
PR	Drama, N.G.	28-May-87	a15d	2048	0.1	0.7	0.1	7.0	5.1	DOB	Neg	Neg	Neg
PR	Drama, N.G.	4-June-87	a20d	512	0.2	1.0	0.0	6.7	4.7				
MO	Ioannina, W.G.	28-Jul-87	a8d	256	0.2	–0.1	0.0	1.2	0.2	Neg	Neg		Neg
MO	Ioannina, W.G.	28-Aug-87	c39d	256	0.0	0.1	0.0	1.6	0.0				
SZ	Ioannina, W.G.	9-Oct-87	a10d	256	0.0	0.1	–0.1	5.6	0.1	DOB	DOB		Neg
EA	Konitsa, W.G.	11-Oct-87	a7d	1024	0.1	0.6	0.0	5.9	0.2	DOB	DOB		Neg
PT	Ioannina, W.G.	11-Oct-87	c4y	Neg	3.5	4.9	0.7	0.0	0.0				
TP	Arta, W.G.	15-Nov-88	c60d	1024	0.3	0.9	0.0	5.4	0.9	Neg	Neg		Neg
TP	Arta, W.G.	12-Apr-89	c5m	Neg	2.6	5.1	0.3	0.5	–0.1				
TP	Arta, W.G.	14-Oct-89	c9m	Neg	1.3	2.1	0.0	5.2	0.1				
SK	Ioannina, W.G.	??-??-89	a	256	0.5	1.5	0.0	6.1	0.7	Neg	Neg		Neg
SK	Ioannina, W.G.	??-??-89	a	256	0.6	1.4	–0.1	6.2	0.5				
SK	Ioannina, W.G.	??-??-89	a	256	0.2	0.8	–0.1	2.0	0.0				
CI	Ioannina, W.G.	7-Jul-89	a6d	4096	0.2	0.2	0.0	5.8	0.1				
CI	Ioannina, W.G.	20-Jul-89	c26d	1024	1.8	1.0	0.0	6.2	–0.1	Neg	Neg		Neg
CI	Ioannina, W.G.	6-Sep-89	c2m	256	2.1	2.5	0.1	1.5	0.0				
HA	Kastoria, W.G.	13-Jul-89	a4d	64	0.1	0.1	0.0	5.7	0.1	DOB	DOB		DOB
PI	Karditsa, C.G.	3-Aug-89	a8d	64	0.4	1.8	0.0	5.2	0.1	Neg	Neg		Neg
PI	Karditsa, C.G.	10-Aug-89	a15d	256	1.0	2.5	0.1	5.1	0.2				
KM	Thesprotia, W.G.	6-Sep-89	c35d	64	1.3	2.2	0.0	1.5	0.0				
GA	Ioannina, W.G.	12-May-90	a	256	–0.2	0.1	0.0	3.3	0.0	DOB	DOB		DOB
SO	Pindus, W.G.	1-Jun-95	a23d	256	0.5	1.3	–0.1	4.2	0.0	Neg	Neg		Neg
GAZ	Kavala, N.G.	10-Jun-95	a	256	0.1	0.1	0.3	2.3	4.6	Neg	Neg	Neg	Neg
KA	Veroia, N.G.	24-Jul-95	a	512	1.1	1.7	–0.1	6.3	1.5	Neg	Neg		Neg
ZI	Kavala, N.G.	21-Aug-95	a	256	3.1	3.9	0.0	5.5	0.1	Neg	Neg		Neg
KAR	Hospital, N.G.	6-Oct-95	a	Neg	0.2	0.5	–0.1	3.9	0.1	Neg	Neg		Neg
KAR	Hospital, N.G.	10-Oct-95	a	128	0.1	0.3	–0.1	2.3	0.0				
SID	Ioannina, W.G.	14-Nov-95	a	64	0.2	0.9	0.1	5.8	0.1	Neg	Neg		Neg
SID	Ioannina, W.G.	24-Nov-95	c	128	0.3	1.4	0.0	6.1	0.0				
GAK	Hospital, N.G.	21-May-96	a	64	1.0	2.5	0.0	6.0	0.0	Neg	Neg		Neg
KO	Hospital, N.G.	5-Feb-96	a	256	0.4	2.5	0.1	7.2	0.4	Neg	Neg		Neg
PA	Veroia, N.G.	12-Aug-96	a10d	256	0.2	1.4	–0.1	6.9	0.1	DOB	Neg		Neg

[a]Number of days (d), months (m) or years (y) after onset of illnes is indicated. Acute (a) and convalescent (c) samples are also indicated.
[b]Adjusted sum OD_{410} values are shown for each specific ELISA test.
[c]Results for each of the nested RT-PCR assays are indicated. Neg denotes negative; DOB, positive PCR band yielding Dobrava virus nucleotide sequences.

between Porogia virus and prototype Hantaan virus (strain 76–118) was demonstrated by plaque-reduction neutralization test and immunofluorescence assay (IFA) with a panel of monoclonal antibodies [Antoniadis et al., 1987].

The recent development of reverse transcriptase-polymerase chain reaction (RT-PCR) approaches for the detection and genetic typing of hantaviruses [Nichol et al., 1993] allows further examination of the samples from HFRS patients to determine the identity of hantaviruses associated with HFRS patients. Using this method, Antoniadis et al. recently provided the first direct genetic evidence for the association of DOB virus with HFRS in two patients from Greece and Albania [Antoniadis et al., 1996]. The purpose of this study was to determine the identity and genetic relatedness of hantaviruses responsible for HFRS in Greece during the last 17 years.

TABLE II. Primers for Nested RT-PCR Detection of Murinae-Associated Hantaviruses

Primer[a]	Nucleotide sequence, 5′ to 3′	Expected product size, bp
	S-segment/N coding region	
MS120C	GGATGCAGAAAAICAGTATGA	
MS1170R	AGTTGTATICCCATIGATTGT	1051
MS364C	GAIATTGATGAACCTACAG	
MS963R	ACCCAIATTGATGATGGTGA	599
	M-segment/G1 coding region	
MM1470C	CCIGGITTICATGGITGGGC	
MM2029R	CCATGIGCITTITCI(G/T)TCCA	560
MM1674C	TGTGAI(A/G)TITGIAAITAIGAGTGTGA	
MM1990R	TCIG(A/C)TGCI(G/C)TIGCIGCCCA	317

[a]Number indicates genome position in aligned hantavirus sequences; C indicates forward-sense primer; I, inosine; R, reverse-sense primer.

MATERIALS AND METHODS

Samples

Fifty-one acute (drawn 5–24 days after onset of disease) and convalescent (drawn more than 25 days after onset of disease) serum samples from 30 HFRS cases were tested, all of which had previously been diagnosed by indirect immunofluorescence assay (IFA) with fluorescein-labeled goat antihuman immunoglobulin (GIBCO Diagnostics, Madison, WI) on spot slides containing Vero E6 cells (CRL 1586; ATCC, Rockville, MD), with ~50% of the cells infected with 76–118 strain of prototype Hantaan virus. Titers were recorded as the greatest dilution of serum, at which characteristic cytoplasmic immunofluorescence was detected (Table I). Blood samples from two of the patients (PA and GAZ) were also tested. The samples were collected during the last 17 years and were stored at −70°C, although many of them had been thawed and refrozen several times. The patients were 26 men and 4 women aged from 21 to 71 years old (mean age 36; S.D. 13).

Virus Strains

HTN strain 76–118 [Lee et al., 1978], Dobrava [Avsic-Zupanc et al., 1995], and PUU strain Sotkamo [Vapalahti et al., 1992] were used for the antigen preparation for the IgM ELISA. The viruses were propagated in Vero E6 cells (CRL 1586; ATCC) cultivated in Eagle's minimal essential medium (MEM) supplemented with 2% fetal calf serum, 2 mM L-glutamine, 60 μg/ml penicillin, and 100 μg/ml streptomycin.

Antibody Detection

For detection of human IgM antibodies, IgM ELISA was performed as described previously [Duermeyer et al., 1979; Feldmann et al., 1993]. Briefly, microtiter plates were coated with antihuman IgM μ-chain goat antibodies (Biosource International, Deerfield, IL), followed by serum samples at fourfold serial dilutions starting at 1:100. HTN and PUU virus antigen slurry and control antigen slurry (uninfected Vero E6 cells) were added, followed first by hantavirus-specific rabbit antibodies and then by antirabbit-horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaiphersburg, MD). Specific antibody binding was detected by ABTS/peroxidase substrate (Kirkegaard and Perry Laboratories).

For detection of human IgG antibodies, microtiter plates were coated with HTN, DOB, or PUU virus-infected cell lysate. Serum samples at fourfold dilutions starting at 1:100 were added. Specific antibodies were detected by horseradish peroxidase-conjugated mouse antihuman IgG (Accurate Chemicals and Scientific Corporation, Westbury, NY), followed by ABTS/peroxidase substrate (Kirkegaard and Perry Laboratories). Samples with adjusted sum OD_{410} values through the four dilutions of >0.5 and >1.0 were considered positive in the IgM and IgG assays, respectively. These cutoff values were based on previous experience with this assay systems with other hantaviruses.

PCR Amplification and Nucleotide Sequencing

RNA was extracted from a selection of 24 acute and two convalescent-phase serum samples (Table I) and two blood samples (patients PA and GAZ) as described previously [Johnson et al., 1997]. Amplification was performed using four sets of nested primers: two sets designed to detect partial N (MS-N) and partial G1 (MM-G1) coding regions of hantaviruses associated with rodents of the Murinae subfamily (HTN, DOB, SEO), one set previously designed to detect the N coding region of hantaviruses associated with rodents of the Arvicolinae subfamily (PUU, PH) [Bowen et al., 1997], and one set previously designed to detect the G2 coding region of the Murinae-associated hantaviruses [Antoniadis et al., 1996]. The nucleotide sequence of the two sets of newly designed primers, as well the position in the hantavirus genome sequence alignments and the expected size of the amplified PCR product, are presented in Table II. RT-PCR and second-round PCR was performed also as previously described [Johnson et al., 1997]. Amplified products were analyzed by electrophoresis in 2% agarose gels in Tris-acetate buffer. The gel was stained with ethidium bromide and PCR products were visualized by UV transillumination. To obtain sequence data, specific PCR products were purified from gel slices using the Mermaid kit (BIO 101, La Jolla, CA) or the Sephaglas Bandprep kit (Pharmacia Biotech, Piscataway, NJ) ac-

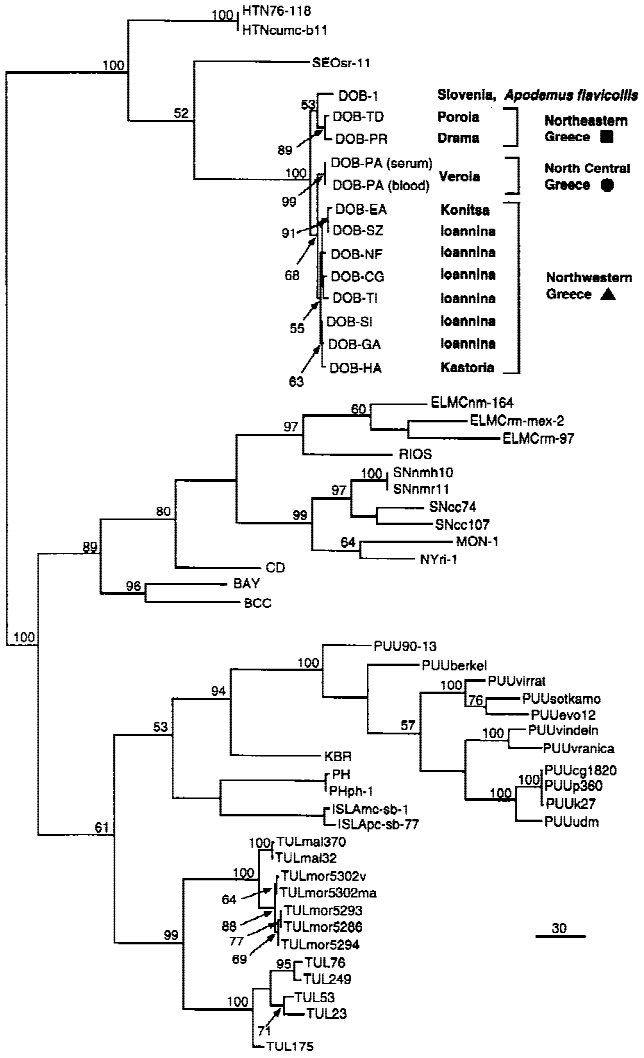

Fig. 1. Virus phylogenetic tree based on nucleotide sequence differences between hantavirus S-segments. Phylogenetic analysis as determined by unweighted maximum parsimony analysis of a 531 nt region of the S-segment of Greek Dobrava (DOB) viruses and other previously characterized hantaviruses. Square, circle, and triangle indicate different geographic groups among the Greek DOB viruses (see also Fig. 2). Maximum parsimony analysis was performed with PAUP 3.1.1 [Swofford, 1991] using the heuristic search option. Three most parsimonious trees were generated, which differed only in the placement of Sin Nombre (SN) virus strains Convict Creek 74 and 107. A representative tree is shown. Bootstrap confidence limits were calculated using 500 repetitions of analysis with values above 50% indicated at nodes. Horizontal distances are proportional to nucleotide step differences (see bar scale). Vertical branches are for visual clarity only. Sequences used in the analysis are from the following sources: Hantaan (HTN) viruses 76-118 (M14626), CUMC-B11 (U37768); Dobrava (DOB) virus, DOB-1 (L41916); Seoul (SEO) virus SR-11 (M34881); El Moro Canyon (ELMC) viruses NM-164 (U11429), RM-Mex-2 (U18099), RM-97 (U11427); Rio Segundo (RIOS) virus RMx-Costa-1 (U18100); Sin Nombre (SN) viruses NM H10 (L25784), NM R11 (L37904), Convict Creek 74 (CC74) (L33816), Convict Creek 107 (CC107) (L33683); Monongahela-1 (MON-1) virus (U32591); New York (NY) virus RI-1 (U09488); Caño Delgadito (CD) virus [Fulhorst et al., 1997]; Bayou (BAY) virus (L36929); Black Creek Canal (BCC) virus (L39949); Puumala (PUU) viruses 90-13 (U22423), Berkel (L36943), Puu/Virrat/25Cg/95 (Z69985), Sotkamo (X61035), Evo/12Cg/93 (Z30702), Puu/Vindeln/L20Cg/83 (Z48586), Vranica (U14137), CG1820 (M32750), p360 (L11347), K27 (L08804), Udmurtia/894Cg/91 (Z21497); Khabarovsk (KBR) virus (U35255); Prospect Hill (PH) viruses PH (M34011), (X55128), PH-1 (Z49098); Isla Vista (ISLA) viruses MC-SB-1 (U31534), PC-SB-77 (U31535); Tula (TUL) viruses Malacky/Ma370/94 (Z68191), Malacky/Ma32/94 (Z48235), Tula/Moravia/5302v/95 (Z69991), Tula/Moravia/5302Ma/94 (Z49915), Tula/Moravia/5293Ma/94 (Z48574), Tula/Moravia/5286Ma/94 (Z48573), Tula/Moravia/5294Ma/94 (Z48741), Tula/76Ma/87 (Z30941), Tula/249Mr/87 (Z30944), Tula/53Ma/87 (Z30942), Tula/23Ma/87 (Z30945), Tula/175Ma/87 (Z30943).

cording to the instructions provided by the manufacturers. Direct sequencing of the purified PCR products was conducted using the dye termination cycle sequencing technique (Applied Biosystems, Foster City, CA) and an ABI 377 sequencer. Sequence compilation and analysis were performed using Sequencher version 3.0 (Gene Codes Corp., Ann Arbor, MI) and the Wisconsin Sequence Analysis Package version 9.0 (Genetics Computer Group, Madison, WI). Phylogenetic analysis of aligned sequences was conducted with PAUP version 3.1.1 Macintosh computer software [Swofford, 1991].

RESULTS

Serological Results by ELISA

No DOB virus-specific high-titer rabbit antibody was available to allow performance of a DOB IgM capture ELISA test. However, extensive cross-reactivity was expected with the HTN antigen. Using IgM ELISA, 35 of 37 serum samples taken from patients during the acute phase of the disease (between day 4 and 24 of illness) were found positive for IgM-specific antibodies to either HTN and PUU antigens or only to HTN (Table I). Two of the patients (CG, NF) continued to have detectable levels of IgM antibodies even a year or more after onset of the disease (1 and 2.5 years, respectively). Cross-reactivity was observed in 12 samples where both HTN and PUU IgM were detected; only one sample (from patient GAZ) had a PUU IgM ELISA optical density value that was significantly higher than that for HTN IgM.

No IgG antibodies to HTN, DOB, or PUU virus antigens were detected in 19 (51%) of the 37 acute serum samples. In addition, 6 of 37 (16%) acute samples were found positive to HTN and DOB, with all but one of these having higher OD values with DOB antigen. Among the acute samples, 8 out of 37 (22%) had detectable IgG reactive only with DOB, 3 (8%) had detectable IgG reactive only with HTN (all from patient NF), and 1 (4%) had detectable IgG reactive with all three antigens (Table I). In the single acute sample (from patient KU) reactive with all three antigens, a higher OD value was observed with the DOB antigen.

Among the convalescent sera, IgG antibodies to HTN, DOB, and PUU were detected in 4 of 14 (28.5%) samples (2 had highest OD values with HTN antigen, 1 with DOB antigen, and 1 with equal OD values for both HTN and DOB). In addition, 6 of 14 (43%) had detectable IgG to HTN and DOB, with all except one having higher OD values with DOB antigen. Single samples were found with IgG only to HTN or DOB antigens, and 2 of 14 (14%) samples contained no detectable IgG to any of the three antigens (Table I).

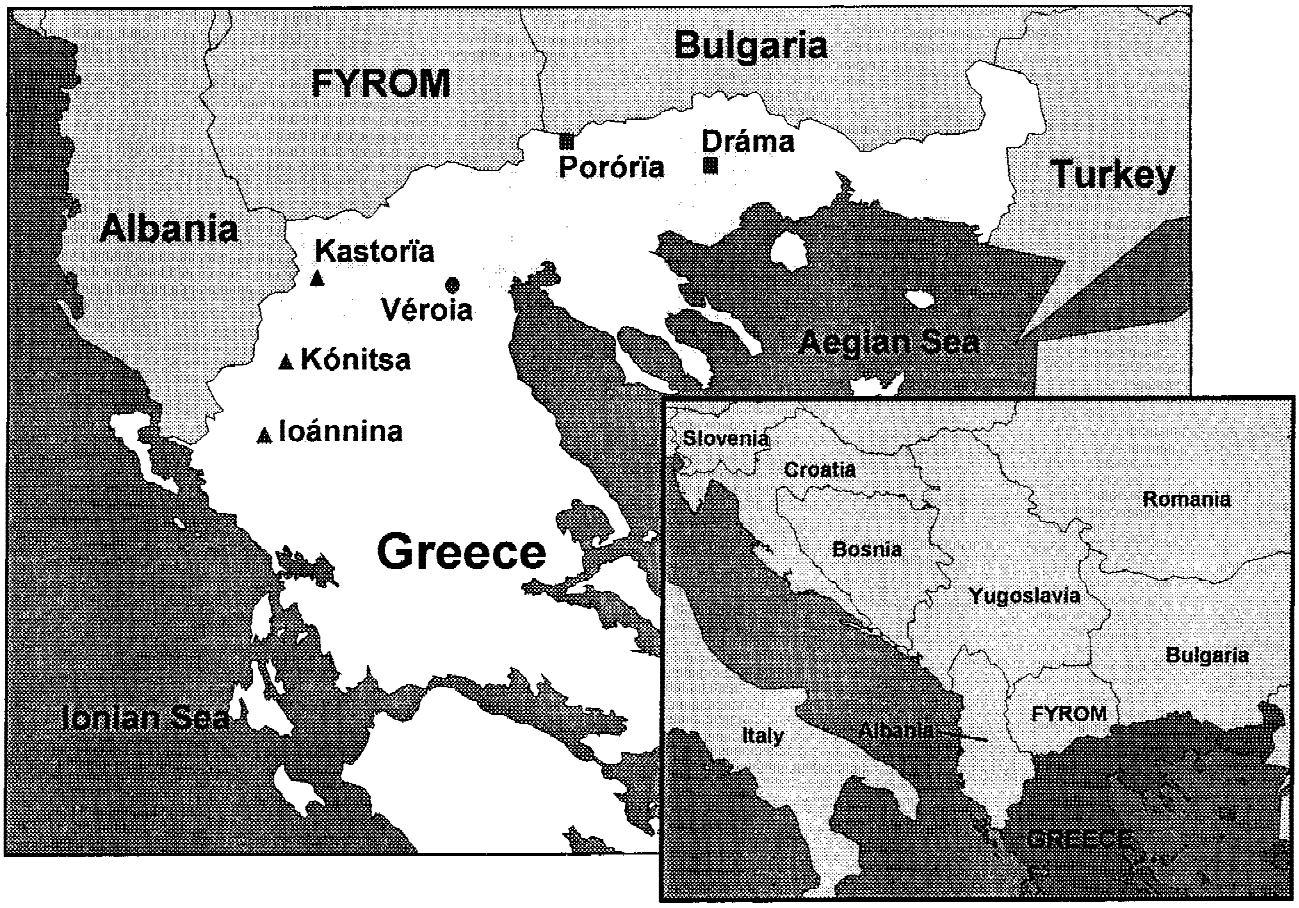

Fig. 2. Geographic location of HFRS cases. The map shows the location of genetically identified HFRS cases in Greece. Symbol shapes correspond to virus genetic lineages identified in Figure 1.

Sequence Analysis

Eleven of the 26 sera tested by PCR produced a band of the expected size (599 bp) using the MS primers, whereas four were positive (band size 317 bp) with the MM primers, and only two with the G2 primers. Whole blood (collected in EDTA) from two of the 26 patients with serum samples was also tested with the same three sets of primers. One whole-blood sample (patient PA) was PCR-positive with the MS primers (equivalent serum sample was also PCR-positive with the MS primers and contained identical sequence) (Fig. 1). The one patient (GAZ) with PCR-negative whole-blood sample also had a negative result with the serum sample.

Nucleotide sequence analysis of the S-segment fragment (517 nt after primer sequences were removed) revealed less than a 5% nucleotide difference among the Greek patients and the previously described Dobrava sequence obtained from *Apodemus flavicollis* captured in Slovenia (DOB-1) (Avsic-Zupanc et al., 1995). At the amino acid level, all the sequences were identical except for DOB-1 and DOB-IT, which shared 96.2% and 99.5% identity, respectively. Phylogenetic analysis of the partial S-segment fragments demonstrated three major lineages among the Greek sequences that were distinguished by geographic location (Figs. 1 and 2).

A 267 nt piece of the G1 coding region of the M-segment contained less than 5% divergence among the four PCR-positive Greek patients. However, when the Greek sequences were compared to DOB-1 and a recently described sequence from a fatal Dobrava human case (DOB-BS) from Slovenia (data not shown), up to 8.6% nucleotide divergence was observed. Amino acid sequences were identical except for DOB-AE (Fig. 3), which contained a change at one amino acid position. When these sequences were subjected to phylogenetic analysis, two major clades were found. The two Slovenian sequences formed one clade and the four Greek sequences constituted the other. All of the Greek sequences originated in the northwestern region of the country.

A small piece of the G2 coding region within the M-segment was sequenced for the two PCR-positive patient samples (HA and GA) and were found to be 99.4% identical to each other. When these sequences were compared to the Slovenian rodent and patient sequences (DOB-1 and DOB-BS, respectively), nucleotide

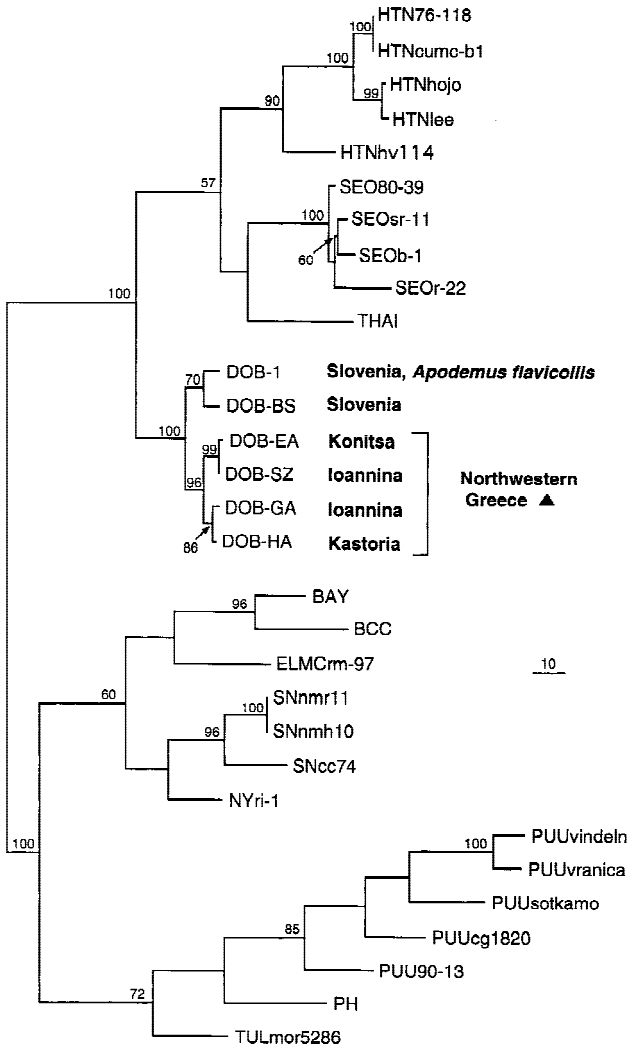

Fig. 3. Virus phylogenetic tree based on nucleotide sequence differences between hantavirus M-segments. Phylogenetic analysis is determined by unweighted maximum parsimony analysis of a 267 nt region of the G1 coding region of the M-segment of Greek Dobrava (DOB) viruses and other previously characterized hantaviruses. Square indicates a particular geographic group among the Greek DOB viruses (see also Fig. 2). Maximum parsimony analysis was performed with PAUP 3.1.1 [Swofford, 1991] using the heuristic search option. Two most parsimonious trees were generated, which differed in the placement of Puumala (PUU) viruses Sotkamo, CG1820, and 90-13. A representative tree is shown. Bootstrap confidence limits were calculated using 500 repetitions of analysis with values above 50% indicated at nodes. Horizontal distances are proportional to nucleotide step differences (see bar scale). Vertical branches are for visual clarity only. Sequences used in the analysis are from the following sources: Hantaan (HTN) viruses 76-118 (M14627), CUMC-B11 (U37729), HoJo (D00376), Lee (D00377), HV114 (L08753); Seoul (SEO) viruses 80-39 (S47716), Sapporo rat (SR-11) (M34882), B-1 (X53861), R22 (S68035); Thailand (THAI) virus Thai749 (L08756); Dobrava (DOB) viruses, DOB-1 (L33685), DOB-BS (data not shown); Bayou (BAY) virus (L36930); Black Creek Canal (BCC) virus (L39950); El Moro Canyon (ELMC) virus RM-97 (U26828); Sin Nombre (SN) viruses NM R11 (L37903), NM H10 (L25783), Convict Creek 74 (CC74) (L33684); New York (NY) virus RI-1 (U36801); Puumala (PUU) viruses Puu/Vindeln/L20Cg/83 (Z49214), Vranica (U14136), Sotkamo (X61034), CG1820 (M29979), 90-13 (U22418); Prospect Hill (PH) virus (X55129); Tula (TULA) virus Tula/Moravia/5286Ma/94 (Z66538).

differences between 9.3% and 10.6% were seen. Dobrava sequences from a Greek (DOB-Nevrokopi-B) and Albanian (DOB-Pindos) patient were recently described [Antoniadis et al., 1996]. A high degree of nucleotide identity was observed between the HA and GA patient sequences and the DOB-Pindos sequence (98.8–99.4%), while a lesser degree of identity was observed with the DOB-Nevrokopi-B sequence (91.9–92.6%). An additional Dobrava sequence was generated from an *A. flavicollis* captured in the same area of Greece as Nevrokopi-B. This sequence was 98.3% identical to Nevrokopi-B at the nucleotide level and between 90.1% and 90.7% identical to the other Greek patient sequences. Finally, a recently described *A. flavicollis* sequence from Bosnia-Herzegovina [Lundkvist et al., 1997] differs from the Greek DOB sequences (including DOB-Pindos) by 5.0–11.2%. Comparison of the Bosnia-Herzegovina nucleotide sequence to the Slovenian sequences reveals a difference of only 1.2–3.7%. The deduced amino acid sequences of all the Dobrava virus G2 sequence fragments are identical.

DISCUSSION

HFRS is endemic in Balkans, where epidemic outbreaks, as well as isolated cases, have been reported during the last two decades. As the rodent hosts for DOB, PUU, and HTN (*Apodemus flavicollis, Clethrionomys glareolus,* and *A. agrarius,* respectively) can be found in the Balkans, it is possible that at least three distinct serotypes (DOB, PUU, and HTN) could be present in this area and be associated with HFRS cases. HFRS cases in the region can generally be classified into two forms: mild HFRS with a mortality rate of <1% and a severe form with a mortality rate of ⩾10%.

In Greece, HFRS is endemic and usually appears in the severe form, similar to that seen in Asia, with symptoms including abrupt onset, high fever, marked thrombocytopenia, flushing over the face and neck, conjuctival injection, and high incidence of severe systematic manifestations, requiring intensive care treatment [Papadimitriou and Antoniadis, 1994]. Serological and genetic analysis of small rodents captured in the endemic areas indicates that *A. flavicollis* is likely to be the principal host of DOB virus in Greece (data not shown) and the first genetic evidence for the association between DOB virus and severe HFRS was demonstrated by RT-PCR using RNA extracted from whole-blood samples of a Greek and an Albanian HFRS patient. In addition, in 1987, an HTN-like virus, Porogia virus, was isolated from the urine of a Greek patient, giving extensive cross-reactivity with HTN 76-118 by IFA with a panel of Mabs and plaque reduction neutralization tests [Antoniadis et al., 1987]. However, PCR analysis performed in 1996 using the earliest available stock of Porogia virus revealed that this virus is indistinguishable from the prototype Korean strain HTN 76-118. At this point, we are unable to confirm the presence of any HTN-like virus in Greece.

For this retrospective study, samples from severely

ill HFRS patients (4 of them died) have been tested. These samples have been taken during the acute and convalescent phase of the disease as well as after a long period after recovery. The serological results by ELISA were found compatible with those of IFA used for the primary laboratory diagnosis. Serology based on IgM ELISA has been proved most efficient for rapid diagnosis of HFRS in all patients using HTN antigen, which cross-reacts highly with DOB antigen used in this study. The significant serological cross-reactivity of HTN, DOB, and (to a less extent) PUU serotype viruses makes specific serotyping of the exact etiological agent difficult. In one patient (NF), the IgG-specific antibodies were higher to HTN than to DOB, even 32 months after onset of the disease, even though the infection was caused by DOB virus, as determined PCR and nucleotide sequence analysis. In the same case, specific HTN IgM antibody were detected even after 32 months from the onset of the disease. To date, HFRS due to PUU virus has not been reported in Greece. The serological results presented here are compatible with all the patients having been infected with DOB virus, with the exception of one. Patient GAZ was possibly infected with PUU virus, as higher IgM ELISA OD values to PUU than to HTN and DOB viruses were detected in the acute serum sample from this patient. However, this sample was negative by PCR analysis, so further studies will be necessary to confirm whether PUU virus infection of humans occurs in Greece.

Based on earlier work, serum samples would be expected to be suboptimal for PCR analysis of hantavirus-infected patients, greater success usually being achieved with whole-blood or frozen tissue samples [Hjelle et al., 1994]. In addition, many of the sera analyzed in this study had been thawed and refrozen several times, which would likely have reduced the quality of the virus RNA present. Given these conditions, the newly designed primer sets (MS-N and MM-G1) targeting hantaviruses associated with the Murinae subfamily rodents performed well on these samples, and should be valuable in the analysis of other patient or rodent materials infected with DOB, HTN, or SEO viruses.

The nucleotide sequencing and phylogenetic analysis reported here shows that there is considerable genetic variation among DOB viruses found throughout the Balkans, and even among viruses maintained in different regions of Greece. Based on the identification of the northwestern, north Central, and northeastern virus genetic lineages, at least three distinct endemic foci appear to be maintained in northern Greece (Fig. 2). While we were not able to confirm the exact identity of the virus associated with all the patients analyzed, the combination of serological and genetic data present would suggest that most of the HFRS cases in Greece are likely associated with DOB virus infections. Four of the 30 HFRS cases analyzed died, and several of the others were severely ill, providing further evidence that DOB virus can cause severe HFRS.

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